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## **Earthworm communities and microbial metabolic activity and diversity under conventional, feed and biogas cropping systems as affected by tillage practices**

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4 **Earthworm communities and microbial metabolic activity and diversity**  
5 **under conventional, feed and biogas cropping systems as affected by**  
6 **tillage practices**

7  
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17  
18 **Highlights**

- 19 • Tillage overwrote the effect of cropping systems on microbial metabolic activity and diversity
- 20 • Tillage overwrote the effect of cropping systems on earthworm communities
- 21 • No-tillage was beneficial for both earthworm communities and microbial activity and diversity
- 22 • Biogas cropping systems with high crop diversity promote soil biota

32 **Abstract**

33 Understanding management factors driving soil biota is pivotal to improve the sustainability of cropping  
34 systems, especially given the emergence of new cropping systems induced by bioeconomy. Here, we  
35 investigated the combined effects of tillage practices (tillage versus no-tillage) and contrasted cropping  
36 systems, including conventional, feed and biogas cropping systems, on earthworm communities and  
37 microbial metabolic activity and diversity (using Biolog Ecoplate). After three years, our results revealed that  
38 tillage overrode the effect of cropping systems on soil biota while being also detrimental for both earthworm  
39 communities and microbial activity and diversity compared with no-tillage. By contrast, no-tillage had  
40 generally a beneficial effect on soil microbial activity and earthworm abundance. More importantly, under  
41 no-tillage, feed and biogas systems increased microbial activity and diversity which was likely due to the  
42 higher crop diversity and the use of digestate instead of manure and slurry compared with the conventional  
43 system. Taken together, our findings show that both earthworm communities and microbial activity and  
44 diversity are very sensitive to tillage. Moreover, microbial activity and diversity are also rapidly affected by  
45 the type of cropping system, suggesting that it would be a better indicator to detect short-term changes in  
46 soil functioning following change in land use. Our study also indicates that, provided that no-tillage is  
47 included, biogas cropping system with high crop diversity can promote soil biota compared with conventional  
48 farming practices, leading likely to the improvement of soil functioning and ecosystem service delivery.

49 **Keywords**

50 Conventional tillage; No-tillage; Soil biodiversity; Soil fauna; Soil microorganisms

## 51           **1. Introduction**

52           Soil biota plays a major role in sustaining soil functions (Wagg et al., 2014). It drives a number of  
53 ecological processes including organic material decomposition, nutrient turnover and energy transfer (Zhang  
54 et al., 2017). Earthworms promote macroporosity by creating underground galleries and improve water  
55 access, nutrient availability and soil aeration (Dulaurent et al., 2020). They are also involved in soil organic  
56 matter (SOM) degradation at several stages, according to their ecological groups, i.e. epigeic, endogeic and  
57 anecic (Huang et al., 2020). Soil microorganisms also mediate many processes that are essential to sustain  
58 soil functions and ecosystem services such as SOM and nutrient recycling, maintenance of soil structure,  
59 reduction of soil erosion, degradation of organic contaminants and the control of pests and diseases  
60 (Kirchman, 2018). Because they respond quickly to change in soil management, both earthworms and  
61 microbial activity have been used as short-term indicators of change in soil functioning and quality (Stone et  
62 al., 2016; Bünemann et al., 2018) and can therefore help in guiding agricultural practices aiming at restoring  
63 soil ecosystem services (Pulleman et al., 2012).

64           It is long known that agricultural practices affect earthworm communities and microbial activity. For  
65 example, earthworm population generally declines following tillage due to mechanical injuries, disruption of  
66 their habitat and modification of their food resources through the inversion of crop residues and the decrease  
67 in available soil water (Ashworth et al., 2017). Tillage practices may also negatively affect microbial activity  
68 by destroying aggregates and habitats and changing environmental conditions such as SOM concentration,  
69 soil moisture and temperature (Roger-Estrade et al., 2010). Although tillage practice is often considered the  
70 main factor affecting soil biota, several studies have shown that cropping systems, rather than tillage  
71 practice, might be the main driver explaining the response of soil organisms to change in land management  
72 (Lopes and Fernandes, 2020). By depositing a more diverse composition of plant residues and root exudates,  
73 crop diversification generally benefits soil organisms diversity and activity (Acosta-Martínez et al., 2007). In  
74 addition, pesticides (Pelosi et al., 2021) and input of organic materials including crop residue, cover crop,  
75 manure, compost and other organic amendments can strongly mediate earthworm population and  
76 communities (Briones and Schmidt, 2017; Leroy et al., 2008).

77 Understanding management factors driving soil biota is needed to improve our capacity to predict  
78 the sustainability of cropping systems, especially given the emergence of new cropping systems induced by  
79 bioeconomy combining food, feed and bioenergy crops (Sheppard et al., 2011). As a low carbon (C) economy,  
80 bioeconomy predominantly relies on biomass which is considered the single source of renewable and natural  
81 C present in enough quantity to be substitutable to fossil fuels and capable of providing human needs, while  
82 storing C to decline global warming (European Commission., 2018). In this context, growing energy-efficient  
83 crops are increasingly integrated to cropping systems in which agro-ecological practices are associated to the  
84 production of biomass for, among others, biogas production (Von Cossel et al., 2019). According to the  
85 seventh European Environmental Action Program, biodiversity conservation and soil protection should be  
86 fully considered in such decisions relating to renewable energy. However, the impact of these innovative  
87 systems (e.g. biogas cropping systems) on soil biology and functioning is poorly documented so far. More  
88 generally, most studies have focused on either the impact of different tillage practices or the effects of  
89 cropping systems on the communities and activities of soil organisms. Whereas it is known that tillage  
90 practice combined with cropping system should be considered a complete system applied in the agricultural  
91 production, few studies have paid attention on the interaction between tillage and cropping system on soil  
92 biota and, ultimately, soil functioning (Zhang et al., 2018). To address this knowledge gap, we investigated  
93 the combined effects of tillage practices and contrasted cropping systems, including biogas cropping system,  
94 on earthworm communities and microbial metabolic activity and diversity.

95

## 96 **2. Materials and Methods**

### 97 *2.1. Study site and experimental design*

98 Our study was carried out on a permanent field experiment established in Beauvais (UniLaSalle farm),  
99 Northern France (49°28'06.6"N 2°04'19.7"E), after wheat crop in 2015. The soil was classified as a Haplic  
100 Luvisol with 14 % sand, 69 % silt and 17 % clay (Gómez-Suárez et al., 2020). Organic C and total N  
101 concentrations were 19.2 g kg<sup>-1</sup> and 0.96 g kg<sup>-1</sup> respectively, and pH was 7.8. The oceanic climate is  
102 characterized by an average precipitation of 655 mm year<sup>-1</sup>. Average minimum and maximum temperatures  
103 vary from 1 to 6.7 °C in winter, 5 to 14.5 °C in spring, 12 to 23 °C in summer and from 7.2 to 15.3 °C in autumn.

104 The field trial started on August 2016 under summer season. Six plots representing two tillage practices and  
105 three cropping systems were studied at this site. Each plot was divided to obtain six replicated subplots. The  
106 size of individual subplots was 24 m x 14 m. The details of each cropping system are shown in Supplementary  
107 Fig S1. Briefly, the conventional cropping system (Conv) was typical of mixed crops and livestock farming in  
108 the region of Picardy (Northern France). The crop rotation was rape/wheat/corn/wheat and the combination  
109 of manure and slurry from cattle was used as soil organic amendment (see Supplementary Table S1 for more  
110 details). The rotation of the transitory system (Feed) was representative of a feed farming system with cover  
111 crops implemented for both forage and biogas production. In this system, crop rotation included 10 crop  
112 species and biogas raw digestate was used as soil organic amendment. The third cropping system aimed at  
113 maximizing the production of biomass for biogas production (Biom). Crop rotation included 12 crop species  
114 consisting mainly in fodder and energy cover crops and biogas raw digestate was used as soil organic  
115 amendment. Treatment frequency index (TFI), which reflects the intensity of pesticide use (Hossard et al.,  
116 2017), is shown for each cropping system in Table S2.

117

## 118 *2.2. Soil sampling and microbial analyses*

119 Soil samples were collected in May 2019 and community-level physiological profiles (CLPPs) based  
120 on the ability of microorganisms to oxidize various substrates were assessed using BIOLOG Ecoplate (BIOLOG  
121 Co., Hayward, CA, USA). In each of the six subplots, five grams of fresh soil from a 2-kg composite soil sample  
122 were collected in each plot and shaken with 45 ml of sterile 0.85% NaCl for 30 min at 200 rpm and then  
123 diluted to 1:1000. Each plate was inoculated with 150  $\mu$ L of the dilution and the plates were incubated at 25  
124 °C. Color development was obtained in terms of optical density (OD) at 590 nm using an automated plate  
125 reader (Varian, Inc., Walnut Creek, CA, USA) (Houben et al., 2020). Average well color development (AWCD)  
126 was calculated as:  $AWCD = \sum(C_i - R)/31$ , where R is the absorbance of the control well (containing water  
127 instead of C source) and  $C_i$  is the absorbance of plate well inoculated with C source  $i$ . Optimal incubation time  
128 was considered at 96h, the most active microbial communities period, as suggested by kinetic curves that  
129 reached the asymptote of color development. This point was therefore considered for further statistical  
130 analyses for each treatment. Richness (S), as the number of oxidized C substrates and the Shannon-Weaver

131 index (H) were calculated using an OD of 0.25 as threshold for positive response. Shannon-Weaver index was  
132 calculated as  $H = -\sum p_i (\ln p_i)$  where  $p_i = (C_i - R) / \sum (C_i - R)$ .

133

### 134 *2.3. Earthworm sampling*

135 Earthworm abundance was assessed in May 2019 using an extraction with formaldehyde solution  
136 coupled with a soil excavation on 20 cm (ISO, 2006). Briefly, in each plot, three extractions were implemented  
137 on 0.7\*0.7 meters quadrats (0.5 m<sup>2</sup>). A solution of 10ml of formaldehyde solution (37%) for 10L of water was  
138 watered 3 times on each quadrat, followed by an observation of 15 minutes. Earthworms were kept in 70%  
139 ethanol for further ecological group (i.e. epigeic, endogeic and anecic) determination (ISO, 2018) based on  
140 morpho-anatomical traits including body length, skin coloration and skin pigmentation. The number of  
141 earthworms was recorded per ecological group for total abundance per plot.

142

### 143 *2.4. Statistical analyses*

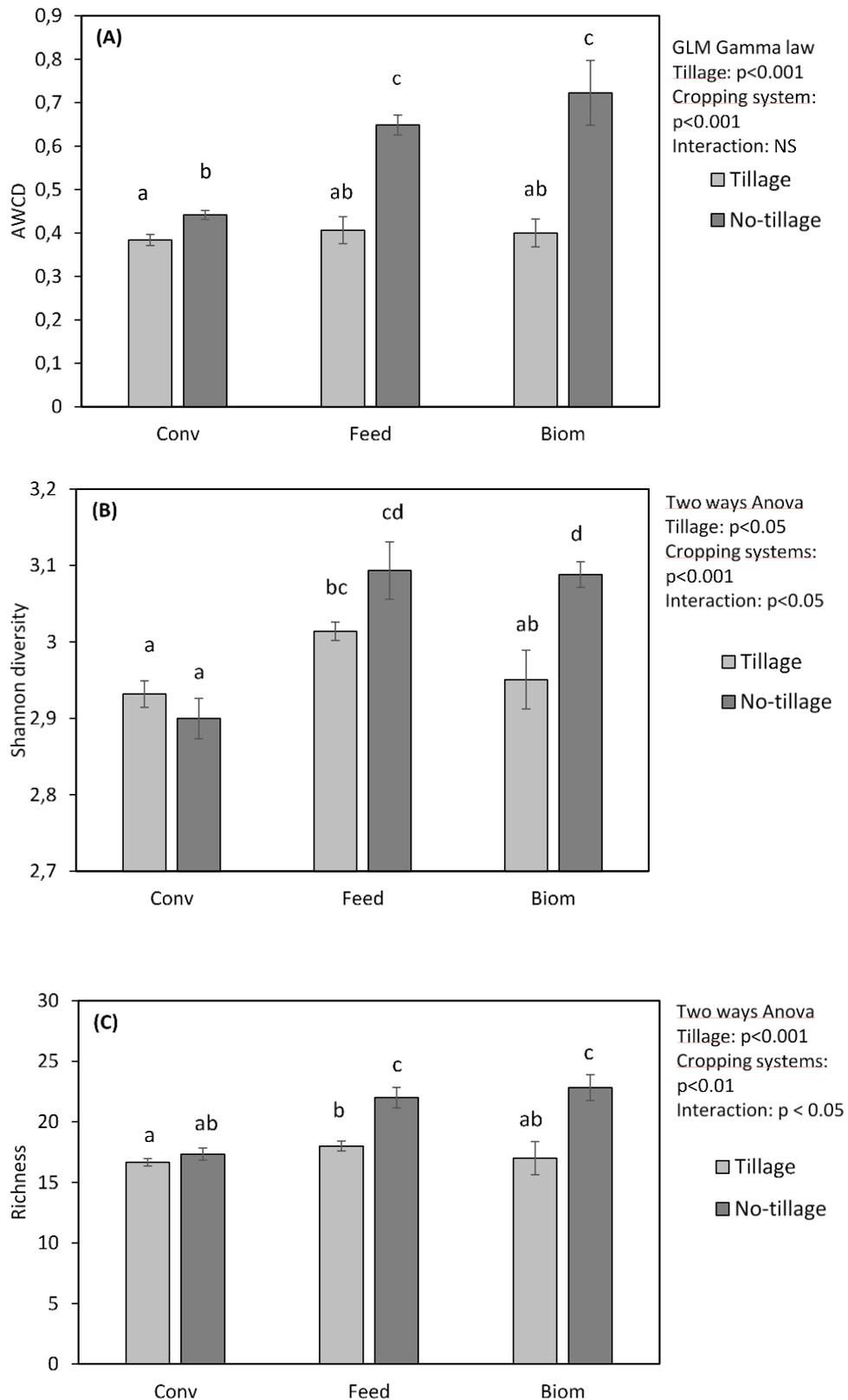
144 Data were analyzed using the statistical software R (version 3.3.2) (R Core Team, 2017). All recorded  
145 data were analyzed using descriptive statistics (mean  $\pm$  standard error) and normality was determined using  
146 the Shapiro-Wilk test. The data were subjected to one-way ANOVA and Tukey's post-hoc test to compare  
147 treatments, which had a normal distribution. Data without normal distribution were subjected to the  
148 Kruskal-Wallis test and Mann-Whitney post-hoc test. In addition, soil enzymatic activity was evaluated via  
149 AWCD, Shannon diversity and Richness indices and considered for two factors: tillage ("Tillage" vs. "No-  
150 tillage") and cropping system ("Conv", "Feed" and "Biom"). Multivariate analyses were based either on two-  
151 ways ANOVA when the normality (Shapiro-wilk test) and heteroscedasticity (Bartlett test) of the variables  
152 was respected, or on Generalized Linear Model (GLM) with the Gamma family using the *fistdistrplus* package  
153 (Delignette-Muller and Dutang, 2015). The same type of tests was performed on earthworm total abundance  
154 and abundances per ecological group (i.e. epigeic, anecic and endogeic).

155

156 **3. Results and Discussion**

157 Our results revealed a strong impact of tillage practices on microbial metabolic activity and diversity  
158 and earthworm communities (Fig. 1 and 2). Unlike no-tillage, tillage overrode the effect of cropping systems  
159 on microbial metabolic activity and diversity since AWCD, Shannon diversity and richness were not  
160 significantly different between the cropping system under tillage (Fig. 1a, b and c). In addition, in the Feed  
161 and Biom cropping system, AWCD, Shannon diversity and richness were much lower ( $p < 0.05$ ) under tillage  
162 than under no-tillage (Fig. 1a, b and c), suggesting that tillage not only overrode the effect of cropping  
163 systems but was also detrimental for microbial activity and diversity compared to no-tillage. Our results were  
164 in agreement with reports from other agrosystems comparing the effect of tillage practices (ploughing,  
165 reduced or no-tillage) on microbial metabolic activity (Sun et al., 2016). In conventional agriculture, tillage  
166 has generally the predominant impact on biological properties since physical disturbance changes soil water  
167 content, temperature, aeration, and the degree of mixing of crop residues within the soil matrix (Lienhard et  
168 al., 2013) while it alters aggregate microhabitat and disturbs fungal hyphal growth (Ashworth et al., 2017).  
169 As a result, most studies report tillage as an abiotic stress in soil biota environment resulting in a decline of  
170 microbial metabolic activity and functional diversity (Govaerts et al., 2007; Houben et al., 2018).

171



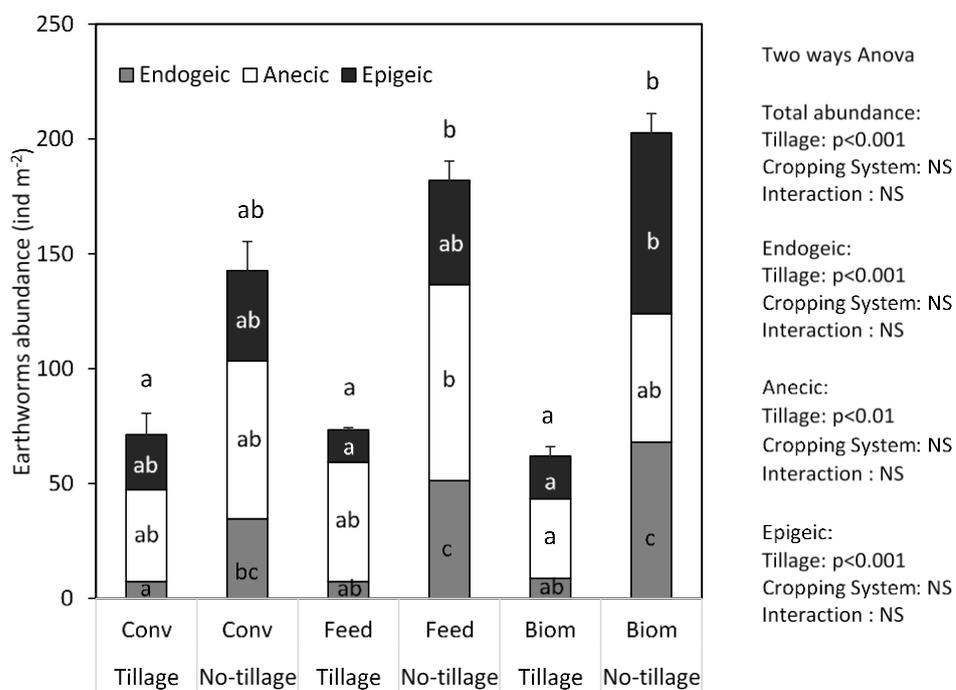
172

173 Fig. 1. Average well color development (AWCD) (A), Shannon diversity Index (B) and Richness (C) in each  
 174 cropping system. Columns with the same letter do not differ significantly at the 5% level (Mann-Whitney  
 175 test for AWCD and Tukey test for Shannon diversity Index and Richness).

176

177 By contrast to cropping systems under tillage, the type of cropping systems had a marked effect on  
178 microbial metabolic activity and diversity under no tillage (Fig. 1a, b and c). The three cropping systems  
179 differed by diverse factors including crop diversity in the rotation (Supplementary Fig. S1) and the type of  
180 organic fertilization (Supplementary Table S1). The Feed and Biom systems were characterized by higher crop  
181 diversity in rotation compared to the Conv one. Under no-tillage, Lienhard et al. (2013) found that crop  
182 diversity was the main factor influencing soil microorganisms. Increasing crop diversity generally stimulates  
183 above-belowground interactions (Zhang et al., 2021) by creating a more habitable resource niche (Zak et al.,  
184 2003). It is therefore likely that the significantly ( $p < 0.05$ ) higher microbial metabolic activity and diversity in  
185 Feed and Biom was due, at least in part, to the higher number of crops in the rotation of these systems.  
186 Overall, our results converge with other works reporting that higher temporal crop diversity provides  
187 belowground benefits including increases of soil microbial metabolic activity, soil microbial biomass and soil  
188 microbial diversity (Acosta-Martínez et al., 2007; McDaniel et al., 2014). High crop diversity was  
189 demonstrated to be the key of bioenergy cropping systems as it favors C sequestration while reducing  
190 agrichemical use (Tilman et al., 2006). Here, our results suggest that implementing crop diversity in biogas  
191 cropping system also supports soil microbial activity and, possibly, soil functioning. In addition, applying  
192 biogas digestate in Feed and Biom instead of manure and slurry might also have increased microbial activity.  
193 Although our understanding of the effects of digestate on soil biota is still incomplete due to their wide  
194 variation in chemical composition (Risberg et al., 2017), our results agree with Walsh et al. (2012) showing  
195 that, due to its higher level of nutrients compared to manure and, especially, slurry (Supplementary Table  
196 S1), digestate might stimulate microbial metabolic activity.

197



198

199 Fig. 2. Total abundance of earthworms and distribution into ecological groups. Columns with the same  
 200 letter do not differ significantly at the 5% level (Tukey test). Standard error and letters above the columns  
 201 account for total abundance.

202

203 Tillage had also a marked effect on earthworms (Fig. 2). Tillage had a detrimental effect on  
 204 earthworms since cropping systems under tillage had generally a significantly ( $p<0.05$ ) lower earthworm  
 205 abundance compared to no-tillage, especially for epigeic and total abundance. These results are in agreement  
 206 with many studies showing that tillage involves a physical perturbation of the tilled layer which leads to a  
 207 disruption of aggregates, causing mortalities, injuries and earthworm locking up in soil clods (Briones and  
 208 Schmidt, 2017). Given the myriad of ecosystem services delivered by earthworms, their decline in tilled  
 209 cropping systems is often associated with undesirable change in the ecological performance of  
 210 agroecosystems (Chan, 2001). The simultaneous increase of earthworm abundance and microbial activity  
 211 under no-tillage is similar to the findings of Kuntz et al. (2013) who concluded that reduced tillage provides  
 212 an approach for eco-intensification of agriculture by not only reducing external inputs but also providing  
 213 additional agroecological services. Although this was not studied in the present study, it must be considered  
 214 that beyond its direct effect on soil biota, no-tillage may also alter trophic interaction, possibly resulting in  
 215 positive feedback between microorganisms and soil fauna (Miura et al., 2008). Unlike microbial activity and

216 diversity, the type of cropping systems had no significant ( $p>0.05$ ) effect neither on total abundance nor on  
217 abundance per ecological group (Fig. 2), suggesting that tillage was the predominant factor affecting  
218 earthworms on the short-term. These findings agree with Simonsen et al. (2010) who concluded that, by  
219 comparing contrasted cropping systems, tillage was the most important factor impacting earthworm  
220 abundance while organic amendment had low effect and crop diversity had no effect. Similarly, Eriksen-  
221 Hamel et al. (2009) also found that no-tillage favored earthworms while cropping management had no  
222 impact, suggesting that physical disturbance, rather than food availability, was the main driver of earthworm  
223 communities. It must however be noted that we analyzed here the relatively short-term response of  
224 earthworms to change in farming practices at only one site. Studies in a longer run and at different places  
225 should be performed to better assess the temporal effect of cropping systems on earthworm communities  
226 while taking into account their spatial variability. For instance, under similar tillage practice, earthworm  
227 abundance is usually positively related to soil organic matter content (Ouellet et al., 2008) even though  
228 negative relationships have also been unexpectedly observed (Bartz et al., 2013). Since change in soil organic  
229 matter content due to e.g. change in crop residue or organic amendment management takes time (Houben  
230 et al., 2018), longer-term studies might possibly reveal different earthworm abundance and communities  
231 between the studied cropping systems. In addition, it has been recently reported that pesticides such as  
232 glyphosate can be chronically toxic to earthworms (García-Pérez et al., 2020), which can alter biodiversity,  
233 hinder recovery, and affect functions (Pelosi et al., 2021). In this study, the relationship between earthworm  
234 abundance and the total use of pesticides in cropping systems was unclear. For instance, irrespective of the  
235 tillage practice, despite a lower use of pesticides (Supplementary Table S2), Conv did not show a higher  
236 earthworm abundance compared to Feed. As demonstrated by García-Pérez et al. (2014), evaluation of the  
237 effects of pesticides is difficult in field studies because their short-term effects can be very subtle and are mixed  
238 with other factors. Therefore, the relative contribution of pesticides on earthworm communities should also  
239 be better elucidated in longer studies in order to optimize the design of innovative cropping systems.

240 Taken together, our findings show that both earthworm communities and microbial metabolic  
241 activity and diversity are very sensitive to tillage. Moreover, microbial metabolic activity and diversity are  
242 also rapidly affected by the type of cropping system, suggesting that it would be a better indicator to detect

243 short-term changes in soil functioning following change in land use. More specifically, our study indicates  
244 that, provided that no-tillage is included, biogas cropping system with high crop diversity can promote soil  
245 biota compared with conventional farming practices.

246

247

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256

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418 **Supplementary Materials**

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420 **Earthworm communities and microbial metabolic activity and diversity under conventional, feed**  
421 **and biogas cropping systems as affected by tillage practices**

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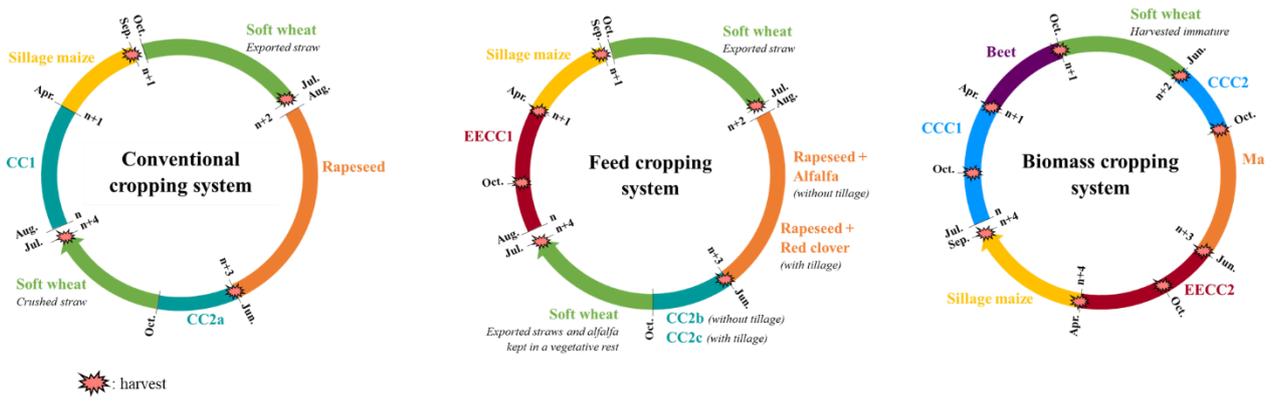
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Cover crop 1 (CC1)	Cover crop 2 (CC2)	Energy-efficient cover crop 1 (EECC1)	Energy-efficient cover crop 2 (EECC2)	Catched cover crop 1 (CCC1)	Catched cover crop 2 (CCC2)	Maslin (Ma)
50% Spring vetch 25% Phacelia 25% Oat	Regrowth of: (a) Rapeseed and alfalfa (b) Rapeseed and alfalfa (c) Rapeseed and red clover	25% Rye 25% Phacelia 20% Italian ray-grass 30% Red clover* 25% Spring vetch* 25% Winter horse bean*	25% Maize 25% Rye 20% Italian ray-grass 25% Winter horse bean* 25% Winter vetch* 30% Red clover*	50% Rye 30% Winter fodder pea 20% Winter horse bean	50% Rapeseed 50% Ryegrass	50% Winter barley 25% Winter fodder pea 25% Winter vetch

\*Harvested in April

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451 **Fig. S1. Crops rotation in Biom, Feed and Conv with or without tillage.**

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471 **Table S1: Rate of application and composition of soil amendment applied onto each cropping.**

Cropping system	Tillage practice	Date	Amendment	Rate of application (m <sup>3</sup> ha <sup>-1</sup> or t ha <sup>-1</sup> *)	N (g kg <sup>-1</sup> )	P <sub>2</sub> O <sub>5</sub> (g kg <sup>-1</sup> )	K <sub>2</sub> O (g kg <sup>-1</sup> )
Conv	No tillage	13/08/2016	Manure	35 *	8.17	5.55	5.41
Conv	Tillage	13/08/2016	Manure	35 *	8.17	5.55	5.41
Conv	No tillage	04/05/2017	Slurry	44	1.8	0.26	1.4
Conv	Tillage	04/05/2017	Slurry	44	1.8	0.26	1.4
Conv	No tillage	31/08/2018	Slurry	24	1.27	0.22	1.39
Conv	Tillage	31/08/2018	Slurry	24	1.27	0.22	1.39
Feed	No tillage	05/08/2016	Digestate	26.5	6.08	1.07	6.18
Feed	Tillage	05/08/2016	Digestate	26.5	6.08	1.07	6.18
Feed	No tillage	10/05/2017	Digestate	26.5	5.24	0.82	4.5
Feed	Tillage	10/05/2017	Digestate	26.5	5.24	0.82	4.5
Feed	No tillage	01/03/2018	Digestate	24.5	3.2	0.51	2.2
Feed	Tillage	01/03/2018	Digestate	24.5	3.2	0.51	2.2
Feed	No tillage	27/07/2018	Digestate	20.5	3.9	0.65	2.9
Feed	Tillage	27/07/2018	Digestate	20.5	3.9	0.65	2.9
Feed	No tillage	16/03/2020	Digestate	20	4.07	1.67	3.97
Feed	Tillage	16/03/2020	Digestate	20	4.07	1.67	3.97
Biom	No tillage	05/08/2016	Digestate	26.5	6.08	1.07	6.18
Biom	Tillage	05/08/2016	Digestate	26.5	6.08	1.07	6.18
Biom	No tillage	01/03/2018	Digestate	24.5	3.2	0.51	2.2
Biom	Tillage	01/03/2018	Digestate	24.5	3.2	0.51	2.2
Biom	No tillage	08/06/2018	Digestate	25	3.47	0.6	2.7
Biom	Tillage	08/06/2018	Digestate	25	3.47	0.6	2.7
Biom	No tillage	22/03/2019	Digestate	22	3.8	1.66	3.69
Biom	Tillage	22/03/2019	Digestate	22	3.8	1.66	3.69

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486 **Table S2: Treatment frequency index (TFI) for each cropping system. Data show total TFI which is the sum**  
 487 **of the TFI for herbicides and the TFI for pesticides other than herbicides.**

Tillage Practice	Cropping system	Year	Total TFI	TFI for herbicides	TFI for others
Tillage	Conv	2017	1.63	1.63	0
		2018	5.9	2.58	3.32
		2019	7.1	1.6	5.5
		Average	4.88	1.94	2.94
Tillage	Feed	2017	1.63	1.63	0
		2018	5.9	2.58	3.32
		2019	14.2	3.2	11
		Average	7.24	2.47	4.77
Tillage	Biom	2017	2.37	1.37	1
		2018	4.5	2.58	1.92
		2019	1	0	1
		Average	2.62	1.32	1.31
No-tillage	Conv	2017	2.23	1.8	0.43
		2018	7.26	2.94	4.32
		2019	8	2.08	5.93
		Average	5.83	2.27	3.56
No-tillage	Feed	2017	2.56	2.13	0.43
		2018	7.26	2.94	4.32
		2019	8	2.08	5.93
		Average	5.94	2.38	3.56
No-tillage	Biom	2017	3.47	2.04	1.43
		2018	5.5	2.58	2.92
		2019	4	1	3
		Average	4.32	1.87	2.45

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